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Fasting can protect young and middle-aged *Drosophila melanogaster* flies against a severe cold stress

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Abstract

Flies were starved with water before being subjected to various severe stresses (heat, cold, fungal infection, hydrogen peroxide) immediately after starvation or after a delay. Starvation of young and middle-aged flies increased resistance to a long cold stress (0°C for up to 48 h), mainly if there was a 2-6 h delay between starvation and the cold stress, but positive effects in old flies were hardly observed. No positive effect was observed on resistance to the other stresses and starvation rather decreased resistance to them. It thus seems that fasting increases frailty but also puts at play mechanisms increasing resistance to cold. Starvation also increased learning scores but this could be linked to decreased positive phototaxis tendencies, and not to a better learning ability. Starvation appears to be a mild stress with limited hormetic effects, but studying the mechanisms of these effects is of interest because fasting is maybe of therapeutic value in human beings.

Key-words

Fasting — heat stress — cold stress — oxidative stress — fungal infection — learning — phototaxis — *Drosophila melanogaster*

Introduction

A mild stress, i.e. a stimulus disturbing the homeostasis of the organism without inducing severe damages, can provoke an adaptive response enhancing the ability to resist other stresses: this phenomenon is called hormesis (reviews in Mattson and Calabrese 2010). Mild stresses, such as heat, cold and hypergravity (HG) can increase longevity or resistance to severe stresses and improve healthspan in *Drosophila melanogaster* (for a review in various species, see Le Bourg 2009; for heat in *D. melanogaster* see also Lagisz et al. 2013)**, but sex and genetic background can modulate the effects of mild stress on longevity (e.g. Sarup and Loeschcke 2011)**. However, deleterious effects of mild stresses can be observed in female flies, because HG can slightly decrease longevity (Le Bourg et al. 2000) and cold has been observed either to increase (e.g. Le Bourg 2007) or decrease longevity (Le Bourg 2010a) or to be neutral (Le Bourg 2007, 2011). Positive effects of mild stress can be observed at old age, even if the mild stress is applied at various ages (Le Bourg 2011), and the positive effects of two mild stresses, HG and cold, can be additive (Le Bourg 2012). One of the features of hormetic treatments is that a too mild stress cannot give rise to positive effects while severe stresses have deleterious effects, intermediate stresses providing positive effects (Calabrese et al. 2012). In flies, positive effects are observed after a rather short exposure to a mild stress while longer exposures can be detrimental. For instance, keeping male

flies in HG for one week does not increase longevity (Le Bourg et al. 2000) and life-long exposures combined with a high HG level decrease it (Le Bourg and Lints 1989; Lints et al. 1993). In contrast, 2-4 weeks exposures can increase longevity in males (e.g. Le Bourg et al. 2000).

Another treatment, dietary restriction (DR), is considered by many authors as a nearly universal means to increase longevity and improve healthspan (reviews in Everitt et al. 2010), even if it does not seem to increase lifespan in various species and mouse genotypes (reviews in Le Bourg 2010b; Nakagawa et al. 2012; Swindell 2012). However, there are major differences between mild stress and DR. Firstly, mild stresses can increase longevity and severe ones (in duration or intensity) decrease it but, in rodents, DR is more efficient as its duration and the percentage of food reduction increase (Bertrand et al. 1999), provided a malnutrition threshold is not reached (see Fig. 4 in Speakman and Mitchell 2011). Secondly, DR increases mean longevity (up to +50%) and maximal longevity while mild stress only increases mean longevity (+20% at a maximum, see Fig. 1 in Minois 2000). Therefore, DR cannot be considered as a mild stress with hormetic effects, because the features and effects of DR and mild stress are different (discussion in Le Bourg 2009).

Most studies of DR in flies have applied a food reduction along adult life but one could wonder whether a short starvation (or fasting), i.e. the complete absence of food for a limited period, could be considered by the organism as a signal for impaired environmental conditions. In such a case, an appropriate strategy would be to prepare for even worse living conditions by increasing resistance to severe stresses such as heat or cold shocks. In other words, a short starvation could be a stimulus disturbing homeostasis without inducing severe damages, and provoking an adaptive response enhancing the ability to resist other stresses: this is the very definition of a mild stress with hormetic effects. By contrast, a long starvation could put the organism at risk, as expected when a too severe stress is applied.

One could oppose to this rationale that DR in flies can impair resistance to severe stresses. For instance, removing live yeast from the nutritious medium decreases resistance to cold, fungal infection and starvation (Le Rohellec and Le Bourg 2009) and Burger et al. (2007) showed that DR decreased resistance to starvation and oxidative stress and, to a lesser extent, to cold. These studies subjected flies to DR for life and not to starvation for a short period but Vigne et al. (2009) showed that feeding young flies on a life-shortening poor medium for 2 days before an anoxia followed by reoxygenation (this is similar to a cardiac ischemia-reperfusion insult in mammals) strongly increased survival to this stress.

Starving wild-type flies for 24 h induced the expression of the anti-gram-negative-bacterial gene *Diptericin* and of the antifungal gene *Drosomycin* (Brown et al. 2009). These authors showed that, via nitric oxide release (which is active against gram-negative bacteria: Foley and O'Farrell

2003), starvation protected *relish* flies against gram-negative bacteria despite the fact that the Imd pathway protecting against these bacteria is deficient in this mutant. Starvation also stimulated the Toll pathway protecting against gram-positive bacteria and fungi, which culminates in the translocation to the nucleus of the NF-κB-like factor DIF and the synthesis of drosomycin. Thus, starvation did not protect *Dif*^{1} mutants against gram-positive bacteria, because these flies cannot mount an immune response and because nitric oxide does not protect against gram-positive bacteria (Brown et al. 2009). It could therefore be hypothesized that, if starvation would protect wild-type flies against fungal infection, it would not be the case for *Dif*^{1} flies. A 6 h starvation in larvae also induced the expression of antimicrobial peptide genes, particularly *Drosomycin*, but this was not observed in *dFOXO* mutants (Becker et al. 2010). Feeding 2-day-old adult flies for 4 days with sucrose only, which is not starvation however, induced the translocation in the nucleus of the transcription factor dFOXO but, here again, *dFOXO* mutants were unable to display this response (Puig and Tjian 2005, for a review on the links between dFOXO and stress resistance, see Puig and Mattila 2011).

All these results allow suspecting that starvation could increase resistance of flies to fungal infection and other severe stresses. Positive effects of short starvation do also exist in rodents because fasting mice for 3 days (with water ad libitum) or spending 6 days on a protein-free diet strongly improved survival after renal ischemia-reperfusion injury (Mitchell et al. 2010; Peng et al. 2012). Similarly, fasting rats for 3 days protected against deleterious consequences of cardiac ischemia-reperfusion (Šnorek et al. 2012) and fasting them for 2 days decreased mortality after brain ischemia (Marie et al. 1990). Subjecting mice to every other day feeding for 8 days also increased survival after cecal ligation and puncture, an experimental model of sepsis (Hasegawa et al. 2012). Finally, the possible use of starvation in cancer patients (review in Lee and Longo 2011) and its general clinical relevance (Robertson and Mitchell 2013) have been envisaged.

Therefore, in the present study, wild-type flies of various ages were subjected to a short complete starvation (with water ad libitum) to test whether this could increase resistance to severe stresses (cold, heat, oxidative stress and fungal infection). The effect of starvation on learning to suppress photopositive tendencies and on phototaxis was also observed in young flies because a previous study has shown that a mild stress, a cold pretreatment, had some effects on these traits (Le Bourg 2007).

Material and methods

Flies

The wild strain Meyzieu caught at the end of the 1970s in France, near the city of Lyon, was maintained by mass-mating in bottles. Flies were fed on a medium (agar, sugar, corn meal and killed yeast) containing a mold inhibitor (para-hydroxymethyl-benzoic acid) and enriched with live yeast at the surface of the medium.

In order to obtain the parents of the experimental flies, flies laid eggs for one night in a bottle. About 50 pairs emerging from this bottle 9-10 days after egg-laying were transferred to bottles (ca 25 pairs in a bottle): these flies are the parents of the experimental flies. Experimental flies were obtained as follows: eggs laid by ca 5 day-old parents during a ca 15 h period on a Petri dish containing the medium colored with charcoal and a drop of live yeast were transferred by batches of 25 into 80 ml glass vials. At emergence, virgin flies with a duration of preimaginal development of 9-10 days were transferred under ether anesthesia in groups of 15 flies of the same sex to 20 ml polystyrene vials containing ca 5 ml of the medium described above. In the following, the date of emergence is indicated by the number of the week in the calendar year (e.g. the first week of 2012 is 1/2012).

Flies spent their life in an incubator and were transferred to new vials twice a week; the rearing temperature was 25 ± 0.5° C; light was on from 07.00 to 19.00 h (fluorescent lamp).

Starvation procedure

Flies were transferred from their vials to empty 19 ml Falcon 2045 vials (16 × 150 mm) for several hours, the duration being depending on experiments and the plug containing absorbent cotton with distilled water to prevent desiccation. After that, flies were transferred back to their vials if there was a delay before being subjected to the experiments described below, or immediately subjected to these experiments if there was no delay after starvation. At young age, less than ca 1% of flies were observed to die during starvation.

Resistance to cold

Flies were kept in empty polystyrene vials (Falcon 2045) stored in ice at 0° C and, after that, transferred back to their rearing vials at 25° C. The percentage of survivors three days after the cold shock was recorded. This percentage was analyzed with a logistic model testing for the effect of sex and starvation and of their interaction. However, a χ^2 test was used when only

one sex was analyzed.

Resistance to a cold stress at one week of age

The effect of the length of a cold stress (16, 20, 24, 32, 48 or 72 h at 0° C) was studied in a series of successive experiments at 6 days of age in flies not subjected to starvation and in flies starved for 24 h, with no delay after starvation and after various delays (16 h cold stress: 8 h delay, group 13/2012; 20 h cold stress: 2, 4, 8 h delays, group 14/2012; 24 h cold stress: 2, 4, 6 h delays, groups 17/2012 and 07/2013; 24 h cold stress: 6, 24, 48 h delays, group 20/2012; 32 h cold stress: 2, 4, 6 h delays, groups 17/2013; 48 h cold stress: 2, 4, 6 h delays, groups 22/2013; 72 h cold stress: 2, 4, 6 h delays, groups 24/2013).

Resistance to a cold stress at 4 weeks of age

Survival to a 24 h cold stress was observed in 27 day-old flies not subjected to starvation and in flies starved for 24 h (group 19/2012), either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. As many males did not survive the starvation treatment and no one survived to the cold stress, a 20 h starvation period and a 20 h cold stress were used in a new experiment (group 23/2012). As only a few males survived to this cold stress and many ones died during the starvation period, a 16 h starvation period and a 16 h cold stress were used in a new experiment (group 24/2012).

Resistance to a cold stress at 6 weeks of age

Flies were subjected at 41 days of age to a 20 h starvation and to a 20 h cold stress (group 18/2012), either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. As nearly no fly survived to this cold stress, a 20 h starvation period and a 16 h cold stress were used in a new experiment (group 22/2012). As nearly no male survived to this cold stress, a new experiment used a 16 h starvation period and a 16 h cold stress (group 34/2012). Thereafter, other experiments used a 16 h starvation period and either an 8 h cold stress (group 36/2012), a 6 h cold stress (group 40/2012), or a 4 h cold stress (group 38/2012).

Resistance to heat

Resistance to heat at one week of age

Resistance to heat was observed at 6 days of age in flies not subjected to starvation and in flies starved for 24 h, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. Flies

were transferred just before the heat shock into empty polystyrene vials (Falcon 2045), the plug containing absorbent cotton with distilled water to prevent desiccation, and kept in a water-bath set at 37° C for 90 or 120 minutes (respectively, groups 15/2012 and 16/2012). Thereafter, they were transferred back to their vials and the percentage of survivors one day after the heat shock was recorded. For each heat shock duration, this percentage was analyzed with a logistic model testing for the effect of sex, starvation group, and their interaction. However, in order to take into account the death of flies observed to be moribund one day after the heat shock, survival was also recorded up to 3 days after the heat shock but this did not modify the results of statistical analyses.

Resistance to heat at 4 weeks of age

Resistance to heat (90 min at 37° C) was observed at 27 days of age in flies not subjected to starvation and in flies starved for 16 h, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay (group 39/2012).

Resistance to heat at 6 weeks of age

Resistance to heat (60, 75 or 90 min at 37° C, respectively groups 45/2012, 3/2013 and 42/2012) was observed at 41 days of age in flies not subjected to starvation and in flies starved for 16 h, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay.

Resistance to hydrogen peroxide

Resistance to hydrogen peroxide at one week of age

Flies not subjected to starvation or starved for 24 h were transferred at 6 days of age (group 35/2012), either at the end of starvation (no delay) or after a 2, 4, or 6 h delay, to polystyrene vials (diameter: 17 mm, length: 63 mm) closed by a polypropylene plug, as in a previous article (Le Bourg 2008). This plug was cut with a razor blade in order to insert into it a strip of chromatography paper (Whatman, 3MM Chr, ca. 10 by 30 mm). One hundred µl of a M/2 saccharose solution (Prolabo 27478.296) were deposited on the strip with hydrogen peroxide (3.3% (w/v), i.e. 979 mM) diluted from 30% (w/w) hydrogen peroxide (Prolabo 23622.298). New solutions of saccharose were prepared each week and solutions were stored at 4°C. In order to prevent desiccation, the vials containing the flies were stored in closed wet boxes. Every day and up to the death of the last fly, the number of dead flies was recorded, the plug and the strip were replaced by new ones and 100 µl of the solution were deposited on the new strip. As the plug was

tightly inserted into the vial, the old strips were still wet when they were discarded, i.e. flies were not subjected to desiccation. The survival times were analyzed with a factorial ANOVA testing for the effect of sex, starvation group, and their interaction.

Resistance to hydrogen peroxide at 4 weeks of age

Resistance to hydrogen peroxide was observed at 27 days of age in flies not subjected to starvation and in flies starved for 16 h, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay (group 50/2012). The survival times were log-transformed before computing a factorial ANOVA testing for the effect of sex, starvation group, and their interaction.

Resistance to hydrogen peroxide at 6 weeks of age

Resistance to hydrogen peroxide was observed at 41 days of age (group 52/2012), the very same procedure as that used with 4 week-old flies being used.

Longevity of infected flies

Infection procedure

The spores of the fungus *Beauveria bassiana* kept at -80° C in 20% glycerol were incubated at 25° C in 90 mm Petri dishes containing the appropriate medium (for one liter of distilled water, the autoclaved medium contained: peptone (Sigma P463): 1 g, glucose (Fluka 49159): 20 g, malt extract (Fluka 70167): 20 g, agar: 15 g). After sporulation, which occurs ca 4 weeks after spreading spores on the medium, flies were infected. Flies were very slightly anesthetized with ether and shaken for ca one minute in a Petri dish containing a sporulating fungal culture. After having checked under stereomicroscope that all flies were well covered with spores, flies were transferred to new vials.

Longevity after infection at one week of age

Flies of the group 21/2012 not subjected to starvation or starved for 24 h were infected at 6 days of age, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. Longevity was recorded daily from the day following infection until the death of the last fly. Longevity data were log-transformed before to be analyzed with a factorial ANOVA testing for the effect of sex, starvation group, and their interaction.

Longevity after infection at 4 weeks of age

Flies of the group 43/2012 were infected at 27 days of age, the same procedure as that

used with one week-old flies being used, except that flies were starved for 16 hours.

Longevity after infection at 6 weeks of age

Flies of the group 44/2012 were infected at 41 days of age, the very same procedure as that used with 4 week-old flies being used.

Learning

Individual flies were trained into a T-maze to suppress their natural positive phototactic tendency (Le Bourg and Buecher 2002). Flies had to choose between a lighted arm, leading to a lighted vial containing a filter paper wetted with an aversive quinine solution, and a darkened arm leading to a dry darkened vial (no aversive stimulus). Flies not choosing the lighted vial at the first trial were discarded because they are considered as photonegative. Most of young flies of both sexes have an increased tendency during a 16-trials training session to choose the darkened vial when the lighted vial is associated with aversive stimuli (humidity and quinine, Le Bourg 2005), while most of flies tested with a dry lighted vial repeatedly choose this vial. No effect of age has been observed on this learning task (Le Bourg 2004) and material and methods have been previously described in detail (Le Bourg and Buecher 2002). In the experiments reported below the darkened arm was dry and contained no paper, and the lighted vial contained either a dry paper (Dry group) or a paper wetted with a 10^{-1} M quinine hydrochloride solution (QCl group). The present experiments tested the effect of starvation on learning (QCl group) and phototaxis (Dry group) scores. The 16 trials were divided in 4 blocks of 4 successive trials: choosing the lighted vial was a photopositive choice (score: 1) and choosing the darkened vial was a photonegative choice (score: 0). Thus, flies always choosing the lighted vial got a score of 16.

Various starvation lengths

In a first experiment, one week-old flies were starved or not for various lengths (ca 17.5 to 26 h) before to be trained to test whether starvation could modify learning and phototaxis scores. This experiment was carried out up to obtain 15 flies with intact legs completing the 16 trials for each combination of sex (male or female), starvation (starvation or control) and reinforcement (QCl or Dry) groups (n = 120). Data were analyzed with 4-way repeated measures ANOVAs testing for the effect of sex, starvation and reinforcement groups, and blocks of trials (repeated factor).

Various recovery lengths after starvation

In a second experiment, one week-old flies were starved or not for 24 h before to be transferred to their rearing vials containing the usual rearing medium. They were trained after various delays (ca 0 to 6.5 h) to test whether a delay after starvation could modify the effect of

starvation. This experiment was carried out up to obtain 10 flies with intact legs completing the 16 trials for each combination of sex, starvation and reinforcement groups (n = 80). Data were analyzed with 4-way repeated measures ANOVAs testing for the effect of sex, starvation and reinforcement groups, and blocks of trials (repeated factor).

Results

The results of resistance to stress experiments are summarized in Table 1.

Resistance to cold

Resistance to a cold stress at one week of age

These experiments tested whether a 24 h starvation could increase resistance to a cold stress, and also if the length of this stress (16, 20, 24, 32, 48, or 72 h at 0° C) or the delay after starvation (0, 2, 4, 6, 8, 24, 48 h) had some effect.

Being subjected to starvation increased resistance to a 16 h cold stress (Fig. 1A, $F(2, 433) = 5.91$, $p = 0.0029$) and males better resisted than females ($F(1, 433) = 4.98$, $p = 0.0262$). All male groups and starved females had a ca 90% survival, while not starved (control) females had a ca 50% survival (sex by starvation group interaction: $F(2, 433) = 9.75$, $p < 0.0001$). Thus, starvation increased resistance to a 16 h cold stress in females but this cold stress had nearly no deleterious effect in males. Therefore, a longer starvation was used in the next experiment.

Starvation with a delay before cold stress increased survival to a 20 h cold stress (Fig. 1B, $F(4, 696) = 24.26$, $p < 0.0001$) and no sex effect was observed (F close to 1). The sex by starvation group interaction ($F(4, 696) = 3.24$, $p = 0.0120$) showed that all male groups had a ca 90% survival, except in the no delay group (50% survival). Starved females with a delay before the cold stress had also a ca 90% survival, while control females and starved females with no delay after starvation had a 50% survival. Therefore, starvation increased survival of females, provided there was a delay after starvation, and males with no delay after starvation had a lower survival than the other groups of males, contrarily to what was observed with the 16 h cold stress. Therefore, the 20 h cold stress had more negative effects on survival than the 16 h one, particularly because starvation with no delay before a cold stress decreased survival of males. Starvation with a delay erased these negative effects (males) or increased survival to a cold stress (females).

A 24 h cold stress (Fig. 1C) strongly decreased survival (compare Fig. 1B and C). Starvation increased resistance to cold (Fig. 1C, $F(4, 560) = 17.79$, $p < 0.0001$) and females better resisted than males ($F(1, 560) = 22.81$, $p < 0.0001$). However, the sex by starvation group interaction ($F(4, 560) = 9.52$, $p < 0.0001$) showed that ca 85% of females with a delay before the cold stress but only 40% of females with no delay and nearly no control female survived to cold. In males, starvation had a positive effect if there was a 6 h delay before the cold stress, and the percentage of survivors increased with the length of the delay. As for the 20 h cold stress, starved males with no delay survived less than control males. Thus, a 24 h cold stress is detrimental but flies can be protected if there is a delay after starvation (only with a 6 h delay in males), females with no delay surviving better than control ones, but less than those with a delay. A replicate experiment (group 07/2013) confirmed the positive effect of starvation in females (percentages of survivors ± confidence interval at p = 0.05 of control, no delay, 2, 4 and 6 h delays groups: 52.70 ± 11.37, 82.67 ± 8.57, 100%, 100%, 94.59 ± 5.15, $\chi^2 = 96.99$, 4 df, $p < 0.0001$). In this experiment, the cold stress had nearly no effect in control males and in starved ones with a delay before the cold stress, but it decreased survival if there was no delay (percentages of survivors ± confidence interval at p = 0.05 of control, no delay, 2, 4 and 6 h delays groups: 89.33 ± 6.99, 67.61 ± 10.89, 88.73 ± 7.36, 91.89 ± 6.22, 92.86 ± 6.03, $\chi^2 = 26.18$, 4 df, $p < 0.0001$). The higher resistance to a 24 h cold stress in this replicate experiment prohibits a clear effect of cold to be observed in males, but the effects of starvation in females are similar to those observed in the previous experiment.

A 32 h cold stress (Fig. 1D) strongly decreased survival (compare Fig. 1B–D). Starvation increased resistance to cold (Fig. 1D, $F(4, 720) = 20.11$, $p < 0.0001$) and females better resisted than males ($F(1, 720) = 133.23$, $p < 0.0001$). The sex by starvation group interaction ($F(4, 720) = 5.68$, $p = 0.0002$) showed that no sex effect was observed in control flies but that starved females better resisted than males. In males, starvation had a positive effect only if there was a 4 h delay before the cold stress (post-hoc test).

The 48 h cold stress killed most of the flies (in each starvation and sex group, $70 \leq n \leq 75$). Only a few males survived in the 2 and 6 h delay groups (percentages of survivors ± confidence interval at p = 0.05 of control, no delay, 2, 4 and 6 h delays groups: 0, 0, 1.37 ± 2.67, 0, 11.11 ± 7.26, $\chi^2 = 27.90$, 4 df, $p < 0.0001$). In females, a few flies of the 4 h delay group and ca

one third of the 6 h delay group survived (percentages of survivors ± confidence interval at p = 0.05 of control, no delay, 2, 4 and 6 h delays groups: 0, 0, 0, 5.56 ± 5.29, 31.08 ± 10.55, χ^2 = 78.72, 4 df, $p < 0.0001$). Thus, starved flies survived to a 48 h cold stress only if there was a long delay between starvation and the cold stress and all control flies died. However, a 72 h cold stress killed all flies, even if they were starved before this cold stress (in each starvation and sex group, $67 \leq n \leq 75$, total n = 719).

The effect of long delays after starvation (24 and 48 h) was tested in the next experiment. Survival after a 24 h cold stress differed among the starvation groups (Fig. 1E, $F(4, 539) = 17.45$, $p < 0.0001$). The results of the control, no delay and 6 h delay groups were similar to those previously observed (compare Fig. 1C and E) and survival decreased in the 24 and 48 h groups. The percentages of survival in the 24 h delay groups were similar to those of the control groups, but the 48 h groups had the lowest survival. No sex effect was observed (F close to 1) but the sex by starvation group interaction ($F(4, 539) = 3.06$, $p = 0.0165$) showed that control males survived better than no delay ones, while the contrary was observed in females, as previously observed (compare Fig. 1C and E).

The main conclusion of all these experiments is that a 24 h starvation can increase survival to a severe cold stress in young flies. Survival is the highest if there is a few hours delay between starvation and cold stress, but males with no delay before this cold stress have a lower survival than control males, while females can exhibit the opposite pattern.

Resistance to a cold stress at 4 weeks of age

About one third of the starved 27 day-old males died during the 24 h starvation and, among the survivors, no one survived to the 24 h cold stress. Only two females died during the starvation period (which could be due to natural mortality at this age) and starvation increased their survival (Fig. 2A, $\chi^2 = 13.37$, 4 df, $p = 0.0096$), the highest survival being observed with the longest delay between starvation and cold stress.

Shorter starvation (20 h) and cold stress (20 h) were used in the hope to increase the percentage of survivors. However, ca 23% of the starved males died during the starvation period. No one survived to the cold stress in the no delay and 2 h delay groups, and only a few ones in the other groups (Fig. 2B). Only 2 starved females died during the starvation period and starvation slightly increased survival, provided the delay between starvation and cold stress was 4 or 6 h (Fig. 2B, $\chi^2 = 9.56$, 4 df, $p = 0.0485$).

Therefore, as the percentage of male survivors was still very low, shorter starvation (16 h) and cold stress (16 h) conditions were used in a new experiment. Less than 9% of males and only one female died during the starvation period. Females better resisted than males ($F(1, 556) = 60.74$, $p < 0.0001$) and starvation increased survival, particularly if there was a delay after the starvation period (Fig. 2C, $F(1, 556) = 8.09$, $p < 0.0001$), the interaction between **sex and starvation treatment** being not significant (F close to 1).

On the whole, it can be concluded that starvation at 4 weeks of age had a positive effect on survival to a strong cold stress, provided the starvation and cold periods are shorter than in young flies.

Resistance to a cold stress at 6 weeks of age

Only a few 41 day-old flies died during the 20 h starvation, which could also be due to natural mortality at this age. No male fly survived among the 200 ones subjected to the 20 h cold stress and 3 females survived among the 53 ones subjected to this cold stress. Therefore, the length of starvation is appropriate but the length of the cold stress is too long and it was reduced in the next experiment.

Thus, a 20 h starvation and a 16 h cold stress were used. Less than 10% of males or of females died during the starvation period. Nearly no males survived to the cold stress, except in the groups with a delay after starvation. Starvation increased survival of females, provided there was a delay after the starvation period (Fig. 3A, $\chi^2 = 18.53$, 4 df, $p = 0.0010$).

In a next experiment, a 16 h starvation and a 16 h cold stress were used in the hope to increase survival in males. All females survived starvation but ca 9% of males died. Only one male survived to the cold stress ($n = 239$) and starvation failed to increase survival of females (Fig. 3B, $\chi^2 = 6.72$, 4 df, n.s.), even if there was a tendency for a positive effect to be observed if there was a long delay after starvation.

Therefore, a new experiment used a 16 h starvation and a 8 h cold stress. Only one male and one female died during starvation. Males survived less to cold than females (Fig. 3C, $F(1, 303) = 18.40$, $p < 0.0001$) and, due to a low number of females, both the starvation effect and its interaction with sex were not significant (Fs close to 1), even if starved females tended to better survive than control ones. However, it is clear that there was not any tendency for a positive effect in males.

A new experiment then used a shorter cold stress (6 h) and the same starvation duration (16 h), in the hope to increase survival in males. About 9% of males and 5% of females died during starvation and, as expected, a higher percentage of flies survived to this cold stress, females better surviving than males (66.67 vs 39.91%, $F(1, 420) = 28.64$, $p< 0.0001$). However, starvation and its

interaction with sex had no effect on survival (Fs < 1).

A last experiment then used a still shorter cold stress (4 h) and the same starvation duration (16 h). Only two males died during starvation and a high percentage of flies survived to the cold stress, females better surviving than males (72.50 vs 60.55%, $F(1, 297) = 4.13$, $p = 0.0432$). However, starvation and its interaction with sex had no effect on survival (Fs close to 1).

To sum up all these experiments involving old flies, starvation had a significant positive effect on survival of females to a strong cold stress in one experiment only (Fig. 3A), the same tendency albeit not significant being observed in other experiments. Whatever the strength of the cold stress could be, no positive effect was ever observed in males.

Conclusion

Starvation increased resistance to cold stress at young and middle ages, but no clear effect was observed at old age.

Resistance to heat

Resistance to heat at one week of age

Starvation had some effect in flies heat-stressed for 90 minutes, (Fig. 4A, $F(4, 710) = 5.02$, $p = 0.0005$), flies being subjected to starvation with no delay or a 2 h delay before the heat stress surviving less than the other groups. Males better resisted than females ($F(1, 710) = 51.56$, $p < 0.0001$) and the sex by starvation interaction ($F(4, 710) = 3.12$, $p = 0.0147$) shoved that starved males with no delay before the heat stress better resisted than females.

Sex, starvation and their interaction had no effect in flies heat-stressed for 120 minutes (Fs close to 1), because only 41 of the 728 flies survived (6%).

Therefore, the main conclusion is that starvation had no positive effect on survival to a 90 min heat stress, because survival of starved groups never exceeded that of control flies. In addition, being subjected to starvation with no delay or a short delay before a heat stress was detrimental.

Resistance to heat at 4 weeks of age

Females better resisted than males to a 90 min heat stress (Fig. 4B, $F(1, 392) = 35.23$, $p < 0.0001$). The starvation effect was significant ($F(4, 392) = 7.76$, $p < 0.0001$) as well as its interaction with sex ($F(1, 392) = 6.50$, $p < 0.0001$). Figure 4B shows that starvation had no effect in females while starved males had a lower resistance than control males, this effect being

less important when the delay between the end of the starvation period and the heat stress increased. Thus, no positive effect of starvation was observed and, to the contrary, starvation decreased resistance to heat in males.

Resistance to heat at 6 weeks of age

Only 5 moribund flies survived among the 171 subjected to a 90 min 37° C heat shock. Since starvation did not help old flies to survive this very strong stress, a second experiment used a 60 min 37° C shock. In this experiment, the starvation effect was significant ($F(4, 329) = 13.07$, $p < 0.0001$) as well as its interaction with sex ($F(1, 329) = 4.54$, $p = 0.0014$), but the effect of sex was not significant (F close to 1). Figure 4C shows that starvation had no effect or decreased survival in females while starved males with no delay before heat shock had a lower resistance to heat than control males. By contrast, starved males with a 4 or 6 h delay had a slightly improved survival, a not significant effect however (post-hoc tests) which was mainly due to moribund flies. When these moribund flies had died, 3 days after the heat shock, the percentages of survivors in the control, 4 and 6 h delays male groups were similar (respectively, ca 42, 46 and 47%, these percentages being 42% for the 2 h group and 0% for the no delay group). Thus, starvation decreased resistance to heat if there was no delay between starvation and heat shock and had no effect if there was a delay.

A slightly longer heat stress (75 min) was used in a third experiment. Flies had a slightly lower resistance to heat than in the previous experiment using a 60 min heat shock, but the results were very similar (Fig. 4D; starvation effect: $F(1, 514) = 12.00$, $p < 0.0001$; sex effect: $F<1$; interaction: $F(1, 512) = 3.30$, p 0.0110). Thus, as for the previous experiment, starvation decreased resistance to heat if there was no delay after starvation and had no effect if there was a delay.

Conclusion

Starvation did not increase or decreased resistance to heat stress at all ages.

Resistance to hydrogen peroxide

Resistance to hydrogen peroxide at one week of age

Hydrogen peroxide killed young flies in ca 4 days and males survived very slightly longer than females (means ± SEM: 3.84 ± 0.05 vs 3.47 ± 0.05 days, $F(1, 728) = 28.61$, $p < 0.0001$). Starvation slightly decreased survival time (means ± SEM of control, no delay, 2, 4 and 6 h delays

groups: 3.93 ± 0.07, 3.36 ± 0.08, 3.43 ± 0.08, 3.68 ± 0.08, 3.86 ± 0.08 days, $F(4, 728) = 10.79$, $p < 0.0001$), the interaction with sex being not significant (F close to 1). Therefore, starvation did not help young flies to resist oxidative stress and, to the contrary, slightly decreased resistance.

Resistance to hydrogen peroxide at 4 weeks of age

Females survived one day longer than males (means ± SEM: 3.66 ± 0.07 vs 2.76 ± 0.06 days, $F(1, 505) = 108.96$, $p < 0.0001$). Starvation decreased survival time (means ± SEM of control, no delay, 2, 4 and 6 h delays groups: 3.51 ± 0.11, 3.02 ± 0.14, 2.98 ± 0.11, 3.23 ± 0.11, 3.28 ± 0.10 days, $F(4, 505) = 7.26$, $p < 0.0001$), and the interaction with sex showed that this effect was mainly due to males ($F(4, 505) = 9.72$, $p < 0.0001$). The means of females were in the range 3.31-3.94 days while control males survived for 3.33 ± 0.13 days and the means of starved males were in the range 2.14-2.87 days. Therefore, starvation did not help middle-aged flies to resist oxidative stress and, to the contrary, decreased survival time, mainly in males.

Resistance to hydrogen peroxide at 6 weeks of age

Females survived slightly longer than males (means ± SEM: 3.07 ± 0.09 vs 2.54 ± 0.05 days, $F(1, 316) = 32.79$, $p < 0.0001$). Starvation decreased survival time (means ± SEM of control, no delay, 2, 4 and 6 h delays groups: 3.07 ± 0.11, 2.66 ± 0.11, 2.78 ± 0.11, 2.70 ± 0.10, 2.41 ± 0.09 days, $F(4, 316) = 5.03$, $p = 0.0001$) and the interaction between starvation and sex was not significant ($F < 1$). Therefore, starvation did not help old flies to resist oxidative stress but decreased survival time.

Conclusion

Starvation decreased resistance to hydrogen peroxide at all ages.

Longevity of infected flies

Longevity after infection at one week of age

Males survived longer than females (means ± SEM: 17.76 ± 0.84 vs 9.93 ± 0.26 days, $F(1, 516) = 107.62$, $p < 0.0001$). Most of females died in a narrow range, while some males had a normal longevity, the last one dying more than 60 days after infection. Starvation had no effect on survival time (F close to 1) and the sex by starvation group interaction ($F(4, 516) = 4.17$, $p = 0.0025$) showed that all female groups had similar survival times while males of the no delay and 2 h delay groups survived for a shorter time than the other groups (means ± SEM of the control, no delay, 2, 4 and 6 h delays male groups: 20.43 ± 2.07, 14.63 ± 1.51, 14.61 ± 1.34, 19.02 ± 1.96, 19.96 ± 2.14 days). Therefore, starvation had no positive effect on resistance to

fungal infection in females and decreased survival time of males, this effect being erased if they had a 4 or 6 h delay after starvation before to be infected. However, starved flies did not outlive control ones.

Longevity after infection at 4 weeks of age

Starved flies lived for a shorter time than control ones ($F(4, 556) = 7.50$, $p < 0.0001$, means ± SEM of the control, no delay, 2, 4 and 6 h delays groups: 11.24 ± 0.65, 7.58 ± 0.28, 8.43 ± 0.43, 8.83 ± 0.48, 8.62 ± 0.37 days). The sex factor had no effect on survival time (F close to 1) but its interaction with the starvation factor ($F(4, 556) = 3.98$, $p = 0.0034$) showed that males lived slightly shorter than females in the no delay and 6 h delay groups while they lived slightly longer in the other groups. Therefore, starvation had a negative effect on resistance to fungal infection in middle-aged flies.

Longevity after infection at 6 weeks of age

Females survived longer than males (means ± SEM: 6.35 ± 0.23 and 5.80 ± 0.24 days, $F(1, 346) = 4.48$, $p = 0.0349$). Starvation had no effect on survival time and the sex by starvation group interaction was also not significant (Fs close to 1). Therefore, starvation had no positive effect on resistance to fungal infection in old flies.

Conclusion

Starvation did not increase resistance to fungal infection at all ages, but could decrease it.

Learning

Various starvation lengths

As expected, flies trained with quinine made a higher number of photonegative choices than those trained with a dry vial (Fig. 5, $F(1, 112) = 302.34$, $p < 0.0001$). Starved flies got higher scores than control ones ($F(1, 112) = 10.71$, $p = 0.0014$). The number of photonegative choices increased with the order of blocks ($F(3, 336) = 36.18$, $p < 0.0001$) and the interaction between reinforcement and the order of blocks ($F(3, 336) = 2.67$, $p = 0.0477$) showed that scores of flies trained with quinine reached a plateau (means of the 4 successive blocks: 1.55, 2.35, 2.75, 2.78 photonegative choices), while scores of flies trained with no quinine slightly increased along blocks (0.33, 0.82, 0.95, 1.13 photonegative choices). The sex factor and all the other interactions were not significant, particularly the one between starvation and reinforcement. The starvation effect was thus similar in flies trained with or without quinine, as confirmed by separate ANOVAs showing significant effects

of starvation in each of these two groups (data not shown). The effect of starvation on learning scores is thus linked to a higher tendency to make photonegative choices in the absence of an aversive reinforcer, which prohibits to conclude that starvation simply improved learning scores. Separate analyses also showed that there was no significant correlation between the length of the starvation period and the scores in any sex or reinforcement group (data not shown). The effect of a delay after starvation was studied in the next experiment.

Various recovery lengths after starvation

Flies trained with quinine made a higher number of photonegative choices than those trained with a dry vial ($F(1, 72) = 134.44$, $p < 0.0001$). The number of photonegative choices increased with the order of blocks ($F(3, 216) = 20.01$, $p < 0.0001$) and the interaction between reinforcement and the order of blocks ($F(3, 216) = 6.62$, $p = 0.0003$) showed that scores of flies trained with quinine reached a plateau (means of the 4 blocks: 1.50, 2.53, 2.78, 2.98 photonegative choices), while scores of flies trained with no quinine slightly varied **among** blocks (0.60, 1.03, 0.55, 1.05 photonegative choices). The second-order interaction between sex, reinforcement and blocks was also significant ($F(3, 216) = 2.97$, $p = 0.0328$), mainly because scores of females trained with quinine plateaued while those of males increased with the order of blocks. The sex and starvation factors were not significant, as well as all the other interactions. Separate analyses also showed that there was no significant correlation between the length of the recovery period and the scores in any sex or reinforcement group (data not shown). In summary, when there was a recovery period after starvation, no effect of a 24 h starvation on learning or phototaxis scores was observed.

Conclusion

Starvation seemed to increase learning scores but this effect was due to increased photonegative tendencies. No effect was observed if there was a delay between starvation and training.

Discussion

There is now a large interest for the possible positive effects of dietary restriction on healthspan and lifespan (e.g. Everitt et al. 2010), even if there is a debate on its use in human beings (e.g. Le Bourg and Rattan 2006; Dirks and Leeuwenburgh 2006; Le Bourg 2010b; Gavrilova and Gavrilov 2012). Beside studies on dietary restriction, some results indicate that fasting, i.e. a complete starvation for a short period, can be of therapeutic value (see the introduction and Anton and Leeuwenburgh 2013).

While the results of the very few studies on the effects of fasting in *D. melanogaster* are

promising, because starvation increased resistance to an anoxia-reperfusion injury (Vigne et al. 2009) or protected *relish* flies against gram-negative bacteria (Brown et al. 2009), more studies are needed to know the effects of starvation on resistance to various severe stresses. The present study thus observed resistance to heat, cold, fungal infection, and hydrogen peroxide in wild-type flies. Starvation, with a delay or no delay before the severe stress, did not increase or even decreased resistance to these severe stresses if we except the cold stress. Therefore, starvation increased frailty even if flies had some time to recover after starvation.

Nevertheless, young (Fig. 1) and middle-aged flies (Fig. 2) better resisted to a long 0°C cold stress if, in most of the cases, there was a delay between starvation and the cold stress, but starvation did not clearly increase resistance at 6 weeks of age, except in females of one experiment (Fig. 3A). As starvation often decreased resistance to cold if there was no delay between starvation and the cold stress, it seems that starvation had both positive and negative effects: starvation increased frailty and thus could decrease resistance to cold if this stress occurred with no delay after starvation but, at the same time, starvation induced unknown mechanisms to resist this cold stress. If there was a delay between starvation and the cold stress flies could recover from starvation and take advantage of these mechanisms, which could explain their higher resistance to the cold stress. This higher resistance is maximal 2-6 h after starvation and decreases thereafter (Fig. 1E).

In *D. melanogaster*, not all mild stresses are equally efficient against severe stresses, because hypergravity exposure increases resistance to heat, but has no effect on resistance to cold, hydrogen peroxide or fungal infection, while a cold stress increases resistance to these stresses but marginally to hydrogen peroxide (see table 1 in Le Bourg 2009). A heat stress also increased resistance to cold (Minois 2001), even if a recent meta-analysis showed that it does not increase lifespan (Lagisz et al. 2013), contrarily to pretreatments by cold or hypergravity (Le Bourg 2009). The present results show that starvation is more similar to hypergravity or heat than to cold, because it only increases cold resistance.

What could explain the better resistance of starved flies against cold stress? Starving wild-type flies for 24 h induces the expression of the anti-gram-negative-bacterial gene *Diptericin* and of the antifungal gene *Drosomycin* (Brown et al. 2009). Starving larvae for 6 h also induces the expression of antimicrobial peptide genes, particularly *Drosomycin*, but not in dFOXO mutants (Becker et al. 2010). Therefore, if dFOXO is at play, as expected if starvation occurs (Puig and Mattila 2011), it could be expected that *dFOXO* mutants would not survive better to cold after starvation, contrarily to wild-type flies. In the same way, could it be that *Dif* flies, which are unable to synthesize drosomycin after fungal infection (Rutschmann et al. 2000), could not better survive to a cold stress after starvation? Testing *dFOXO* and *Dif* mutants would be of interest in future

studies. However, it is known that a pretreatment by cold increases resistance to a severe cold stress in *Dif* flies as in wild-type ones (Le Bourg et al. 2012). **It has been shown that a cold-sensitive transient receptor potential channel could partly explain the increased longevity of *Caneorhabditis elegans* nematodes living at colder temperatures, and thus that this increased longevity was not only explained by slower chemical reactions at colder temperatures (Xiao et al. 2013). Could such a phenomenon partly explain the better resistance of flies to cold after being subjected to starvation?**

Learning ability in a T-maze was also studied: a 17-26 h starvation increased learning scores and decreased positive phototaxis tendencies. By contrast, Thimgan et al. (2010), using the same task, did not observe any effect of 7 or 12 h starvations on learning and phototaxis scores. As flies crossing the maze learn to prefer the dark arm of the maze because the lighted one they initially prefer is associated with a punishment, a decreased preference for lighted areas could explain why learning scores increase. Thus, starved flies could choose the darkened arm because starvation decreased their photopositive tendencies, and not because they have a better learning ability or short-term memory. Flies cold-stressed before a learning session using the same task also displayed increased learning scores and decreased positive phototaxis tendencies (Le Bourg 2007). It thus seems that mild stresses, like starvation and cold, can slightly decrease positive phototaxis tendencies. Positive phototaxis tendencies also slightly decrease with age (Le Bourg and Badia 1995) but, as the effect of starvation is reversible, because no effect on phototaxis tendencies and learning scores is observed when there is a delay between starvation and learning (see above), starvation probably does not induce a precocious aging. In the same way, the effects of starvation are probably not explained by an improved memory because a 24 h starvation has no effect on 1-h memory in a pavlovian olfactory conditioning test (Li et al. 2009). Similarly, a 21 h starvation before conditioning with the same olfactory procedure had no effect on memory measured 24 h after conditioning in flies also starved between conditioning and memory testing, thus for a total of 45 h before memory testing (Plaçais and Préat 2013).

In summary, this study shows that fasting can increase resistance to a severe cold stress in *D. melanogaster*, particularly if there is a delay between starvation and the cold stress, but not to several other strong stresses. The positive effect of fasting is thus limited to a few stresses, cold (this study), anoxia-reoxygenation (Vigne et al. 2009) and gram-negative bacterial infection of *relish* flies (Brown et al. 2009), and it can decrease resistance to several severe stresses (heat, fungal infection, hydrogen peroxide: this study), particularly if the stress is applied immediately after starvation.

Yet, fasting has several beneficial effects in mammals (e.g. in the event of cardiac, renal or

624 brain ischemia: see the introduction), and it has been suggested that a longer period of fasting than
625 the current “one-night fast” before surgery could help to protect against post-operative hazards
626 (Mitchell et al. 2010). As fasting for a short period is non-invasive, easy to implement, and a not
627 risky procedure, one could use it before surgery and maybe as an adjuvant to chemotherapy against
628 cancer (Lee and Longo 2011) if it proves to be efficient. Knowing the mechanisms of the protection
629 offered by starvation against cold stress in flies could pave the way for studies in mammals, and
630 maybe in human beings.
631

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635

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Figure captions

Figure 1. Percentage of survivors (± confidence interval at p = 0.05) three days after a long cold stress (0° C) as a function of sex, starvation group, and length of cold stress in 6 day-old flies. The starvation length was always 24 h. On all figures "control" is the no starvation group, and the cold stress was applied at the end of starvation ("no delay" group) or after a delay (2, 4, 6, 8, 24, 48 h groups). A. 16 h cold stress, each bar is the mean of 58-86 flies. B. 20 h cold stress, each bar is the mean of 67-74 flies. C. 24 h cold stress, each bar is the percentage of 54-60 flies. D. 32 h cold stress, each bar is the percentage of 70-75 flies. E. 24 h cold stress, each bar is the percentage of 44-59 flies.

Figure 2. Percentage of survivors (± confidence interval at p = 0.05) three days after a long cold stress (0° C) as a function of sex, starvation group, and length of cold stress in 27 day-old flies. On all figures "control" is the no starvation group, and the cold stress was applied at the end of starvation ("no delay" group) or after a delay (2, 4, 6 h groups). A. 24 h starvation and 24 h cold stress, for each bar n is 44-66 females, 37-72 males were observed in each group but no one survived to the cold stress (ca one third of males died during starvation). B. 20 h starvation and 20 h cold stress, for each bar n is 48-68 females, 26-67 males were observed in each group but no one survived to the cold stress in the no delay and 2 h groups (ca one quarter of males died during starvation). C. 16 h starvation and 16 h cold stress, for each bar n is 35-68 flies.

Figure 3. Percentage of survivors (± confidence interval at p = 0.05) three days after a long cold stress (0° C) as a function of sex, starvation group, and length of cold stress in 41 day-old flies. On all figures "control" is the no starvation group, and the cold stress was applied at the end of starvation ("no delay" group) or after a delay (2, 4, 6 h groups). A. 20 h starvation and 16 h cold stress, for each bar n is 33-44 females, 46-65 males were observed in each group but no one survived to the cold stress in the no delay and 2 h groups (ca 9% of flies of each sex died during starvation). B. 16 h starvation and 16 h cold stress, for each bar n is 19-25 females, 44-57 males were observed in each group but only one survived to the cold stress in the 4 h group (ca 9% of males died during starvation). C. 16 h starvation and 8 h cold stress, for each

773 bar n is 15-23 females or 36-54 males (only one male and one female died during starvation).

774

775 Figure 4. Percentage of survivors (± confidence interval at p = 0.05) one day after a heat
776 shock (37° C) as a function of sex and starvation group. A. 90 min heat stress at 6 days of age,
777 each bar is the percentage of 63-74 flies. B. 90 min heat stress at 27 days of age, each bar is
778 the percentage of 31-51 flies. C. 60 min heat stress at 41 days of age, each bar is the
779 percentage of 25-41 flies. D. 75 min heat stress at 41 days of age, each bar is the percentage of
780 46-59 flies.

781

782 Figure 5. Mean learning (quinine groups) or photonegative (dry groups) scores ± SEM of
783 flies as a function of sex and starvation. The score is the mean number of photonegative choices
784 during 16 trials. Flies were starved or not (ca 17.5 to 26 h). Flies were either trained in a learning
785 procedure (quinine groups) or tested for their photopositive tendency (dry groups). Each bar is the
786 mean of 15 males or females.

Figure
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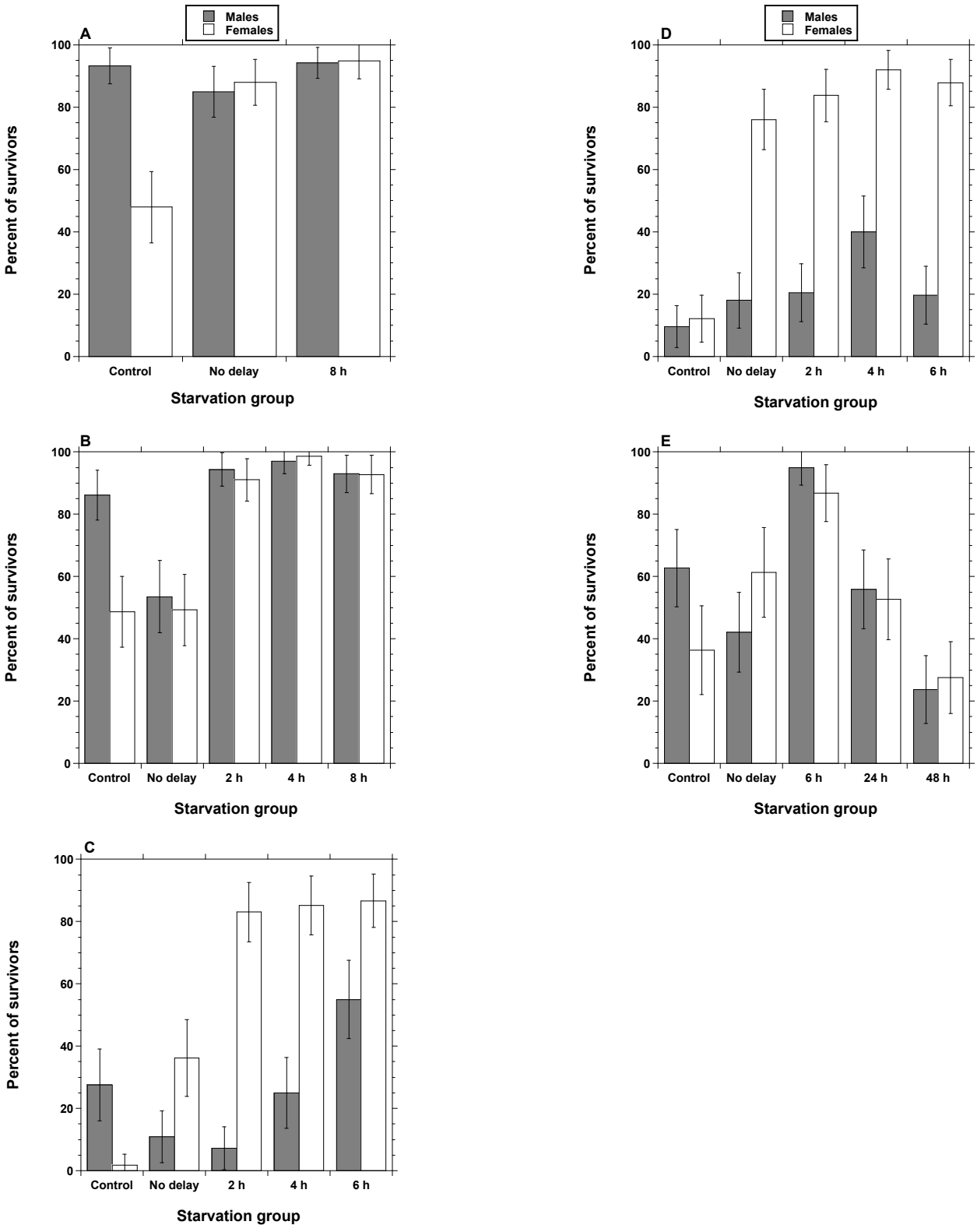

Figure
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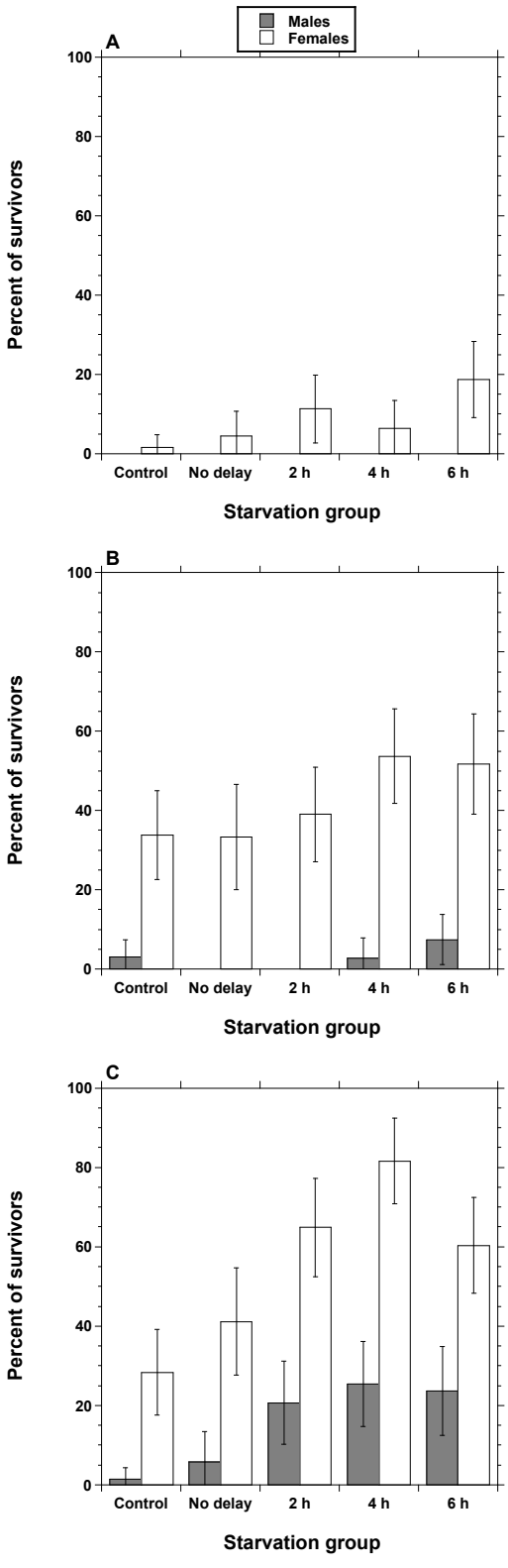

Figure
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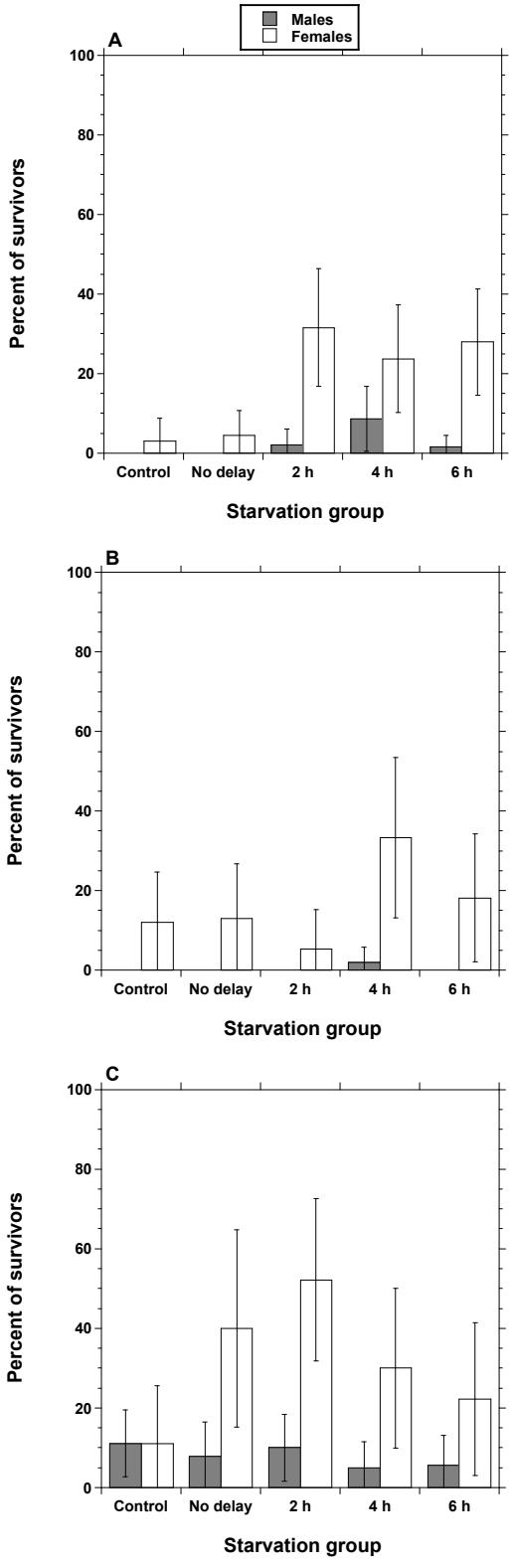

Figure
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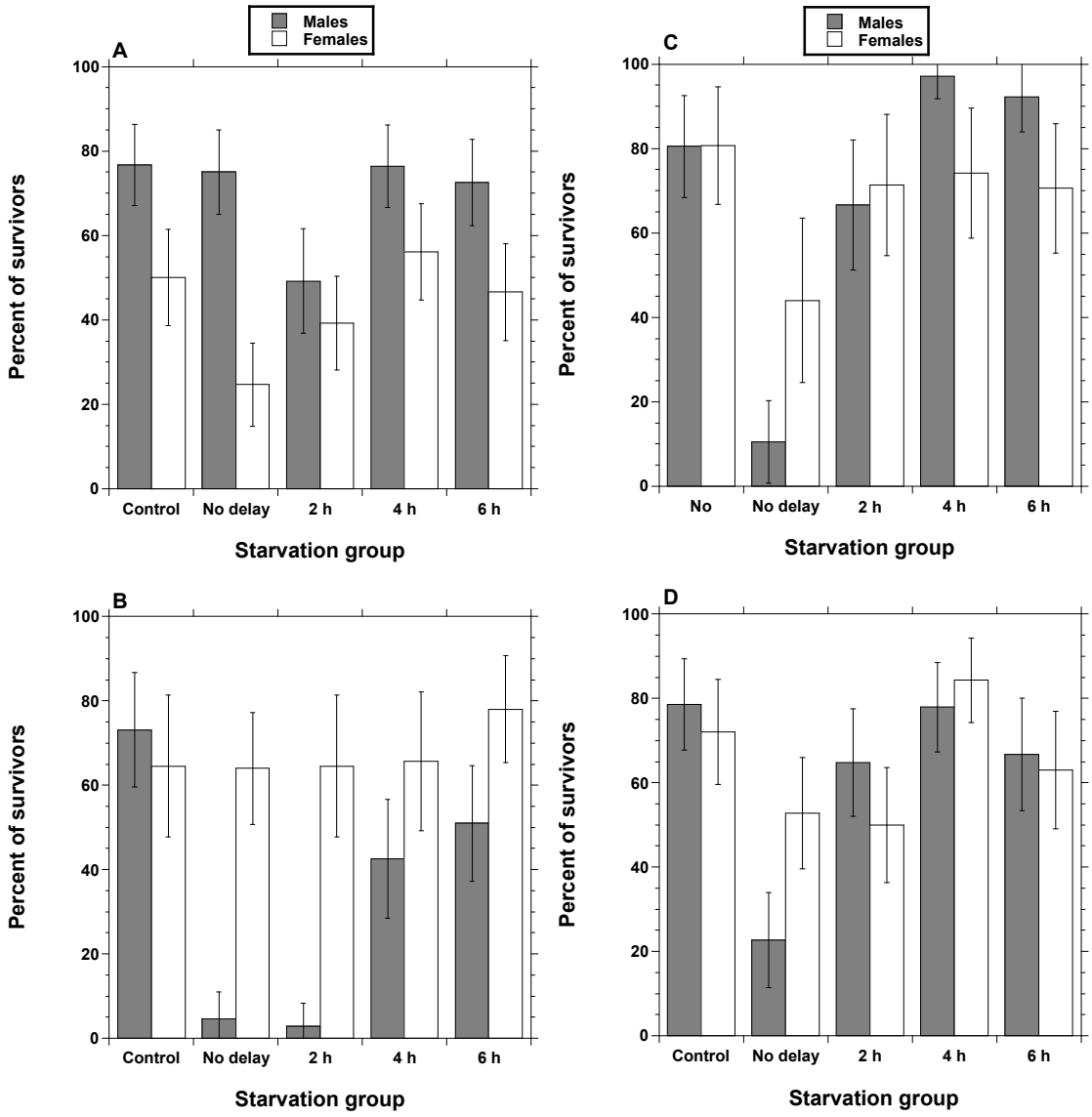

Figure
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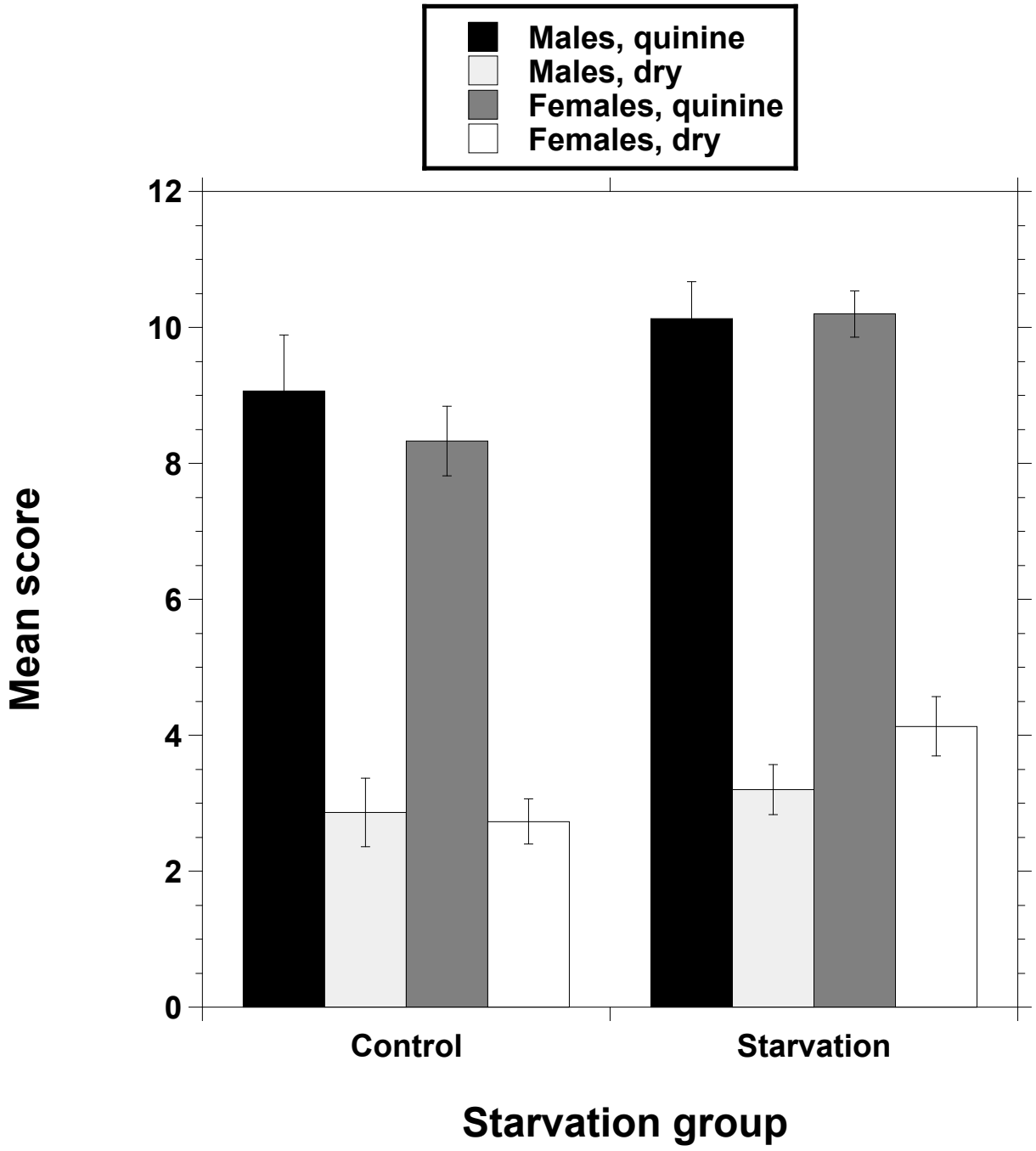

Table
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Table

Table 1. Summary of the effects of starvation on resistance to severe stresses in males and females of various ages. The effect of starvation is shown as 0 (no effect), – (deleterious effect) or + (better resistance to stress). When nearly no fly survived the starvation or stress treatments, this is indicated as "dead". The starvation duration is indicated in parentheses for middle-aged and old flies; it was always 24 h in young flies, but 16, 20, or 24 h in middle-aged and old flies.

	Males			**Females**		
	1 week	4 weeks	6 weeks	1 week	4 weeks	6 weeks
Length of cold stress						
4 h			0 (16)			0 (16)
6 h			0 (16)			0 (16)
8 h			0 (16)			0 (16)
16 h	0	+ (16)	dead (20)	+	+ (16)	+ (20)
16 h			dead (16)			0 (16)
20 h	+	dead (20)	dead (20)	+	+ (20)	dead (20)
24 h	+	dead (24)		+	+ (24)	
32 h	+			+		
48 h	+			+		
Length of heat stress						
60 min			0 (16)			0 (16)
75 min			0 (16)			0 (16)
90 min	–	– (16)	dead (16)	–	0 (16)	dead (16)
120 min	0			0		
Hydrogen peroxide	–	– (16)	– (16)	–	0 (16)	– (16)
Fungal infection	–	– (16)	0 (16)	0	– (16)	0 (16)

Answers to referees

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